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Heterotopic endochondrial ossification with mixed tumor formation in C3(1)/Tag transgenic mice is associated with elevated TGF-beta1 and BMP-2 expression

Ioanna G Maroulakou[1,6], Masa-Aki Shibata[1], Miriam Anver[2], Cheryl L Jorcyk[1,7], Min-ling Liu[1], Nan Roche[1], Anita B Roberts[1], Ilan Tsarfaty[3], James Reseau[4], Jerrold Ward[5] and Jeffrey E Green*[,1]

[1]*Laboratory of Cell Regulation and Carcinogenesis, NCI, Bethesda, Maryland, MD 20892, USA;* [2]*Pathology/Histology Laboratory, SAIC, FCRDC, Frederick, Maryland, MD 21702, USA;* [3]*Sackler School of Medicine, Tel Aviv, Israel;* [4]*ABL, FCRDC, Frederick, Maryland, MD 21702, USA;* [5]*OLAS, NCI, Frederick, Maryland, MD 21702, USA*

Transgenic mice which express the simian virus 40 large T-antigen (Tag) under the regulatory control of the hormone responsive rat C3(1) gene develop unusual lesions of heterotopic bone growth associated with mixed tumor formation arising from eccrine sweat glands found only in the foot pads of mice, ischiocavernosus muscle adjacent to bulbourethral glands and occasionally the salivary and mammary glands. These lesions are very similar to mixed tumors arising in several types of human cancers. Based upon electron microscopic examination and immunocytochemical analyses of cellular differentiation markers, the mixed proliferative lesions in this transgenic mouse model begin with the Tag-induced proliferation of epithelial and myoepithelial cells. The proliferation of these two types of cells results in hyperplasia and adenomatous transformation of the epithelial component, whereas the proliferating myoepithelial cells undergo metaplasia to form chondrocytes which deposit extracellular matrix, including collagen fibers. Cartilage develops focally between areas of epithelial proliferation and subsequently ossifies through a process of endochondrial bone formation. The metaplasia of myoepithelial cells to chondrocytes appears to require the inductive interaction of factors produced by the closely associated proliferating epithelial cells, including members of the TGF-β superfamily. We demonstrate that TGF-beta1 protein accumulates in the extracellular matrix of the lesions, whereas RNA *in situ* hybridization reveals that BMP-2, another strong inducer of heterotopic bone formation, is overexpressed by the proliferating epithelial cells during the development of ectopic bone. The formation of sarcomatous tumors within the mixed tumors appears to be androgen-dependent and more frequent in mice lacking a normal allele of p53. This process of cartilage and bone induction may mimic epithelial-mesenchymal interactions which occur during embryonic bone formation. These transgenic mice may provide new insights into the processes of ectopic endochondrial bone formation associated with mixed tumor formation and serve as a useful model for human heterotopic bone disease.

*Correspondence: JE Green
Current addresses: [6]Medical University of South Carolina, Charleston, SC, USA; [7]Department of Biology, Boise State University, Boise, ID, USA
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Introduction

Transgenic model systems are powerful tools for elucidating relationships between gene expression, development and carcinogenesis (Adams and Cory, 1991; Hanahan, 1988; Merlino, 1994; Jaenisch, 1988). In this report, we present the first transgenic mouse model of heterotopic bone formation associated with the development of mixed tumors.

Mixed tumor formation associated with heterotopic ossification occasionally occurs in human tumors of the salivary, parotid and lacrimal glands (Gnepp, 1993; Ashley, 1990; Jacobson *et al*., 1973; Perzin *et al*., 1980), and the skin (Redono *et al*., 1982). Similar phenomena have been found to occur naturally in other species, such as in canine mammary gland tumors (Moulton, 1978) and in sweat glands of cats and dogs (Jubb *et al*., 1993). However, these animal systems have limited utility in studying molecular mechanisms involved in the early development and progression of these mixed tumors because development of such lesions occurs unpredictably in these species and is diagnosed only at late stages of their development. The transgenic mice described in this report provide a predictable *in vivo* model system for studying early molecular events occurring in the cellular differentiation pathways leading to mixed tumors with ectopic bone formation.

Mixed tumors are derived from cells from more than a single germ layer. They may consist of a mixture of diverse cell types, such as epithelial cells, fibroblasts or other mesenchymal cells and a matrix of fibrous connective tissue, cartilage and/or bone (Moulton, 1978). Heterotopic ossification, often associated with mixed tumors, is characterized by the formation of normal bone at ectopic soft tissue locations and may result from a variety of disorders in the regulation of osteogenesis. There are several rare human genetic diseases of heterotopic ossification (Kaplan *et al*., 1993, 1994; McKusick, 1992; Prakash *et al*., 1989). The ectopic bone formation which occurs in this transgenic model shows some features of several of these disorders including fibrodysplasia ossificans progressiva (FOP) (Kaplan *et al*., 1993), progressive osseous heteroplasia

(POH) (Kaplan *et al*., 1994) and tracheopathia osteoplastica (TPO) (Prakash *et al*., 1989).

Two independent lines of transgenic mice have been studied which express the simian virus 40 large T-antigen (Tag) under the regulatory control of the hormone-responsive rat C3(1) prostatein promoter (Maroulakou *et al*., 1994). This transgene successfully targets the expression of Tag to the mouse prostate epithelium, as originally designed, leading to the development of prostate cancer. However, transgenic mice carrying the C3(1)/Tag transgene also develop mammary cancer and proliferative lesions in other tissues as well (Maroulakou *et al*., 1994; Shibata *et al*., 1998). Both founder lines of these transgenic mice predictably develop unusual mixed proliferative lesions which lead to ectopic bone formation. These unique lesions arise through the transgene-induced proliferation of epithelial cells, particularly in the sweat glands and urethral glands (Shibata *et al*., 1998), but occasionally in the salivary and mammary glands as well. The progression of these lesions results in a cascade of differentiation events affecting both epithelial and mesenchymal elements, leading to ectopic endochondrial bone formation and associated in many cases with mixed tumors. The transforming activity of Tag involves the functional inactivation of the tumor-suppressor genes, p53 and Rb (Chen *et al*., 1992; Ludlow, 1993; Mietz *et al*., 1992; DeCaprio *et al*., 1988; Linzer and Levine, 1979; Lane and Crawford, 1979), which have been implicated in the development of osteosarcomas (Michiels and Merregaert, 1993; Russo *et al*., 1994; Smith-Sorenson *et al*., 1993; Schreck, 1992; Wadayama *et al*., 1993) and chondrosarcomas (Dobashi *et al*., 1993). This transgenic system provides a model in which to study mechanisms for Tag-induced alterations of epithelial and myoepithelial cells which results in heterotopic bone formation.

We have utilized multiple cellular differentiation markers to determine the progression of cellular changes leading to cartilage formation, heterotopic ossification and associated mixed tumor formation. This report demonstrates that ectopic bone formation is associated with the prior overexpression of the osteoinductive factor BMP-2. The roles of p53, sex steroid hormones and two signaling mechanisms important in epithelial cell differentiation (Met and the transforming growth factor beta's) have also been studied during this process. These transgenic mice represent a unique *in vivo* model system to study molecular events involved in heterotopic endochondrial bone formation associated with mixed tumor development.

Results

Establishment of transgenic animals

Six female and six male transgenic founder mice were obtained. Southern blot analysis indicated that they carried from 1 to >100 copies per genome. Multiple, overlapping phenotypic abnormalities were observed in founder animals, all of whom died at 6–20 weeks of age (Maroulakou *et al*., 1994). Six founder animals (three of four females and three of three males) displayed unusual clinical abnormalities in some tissues normally containing cartilage. These animals were identified grossly by the striking, generalized enlargement of the ear pinna. Two of three males developed osteosarcomas in the ossicles of the middle ear or temporal bone. Other phenotypic abnormalities of the founder mice have previously been reported (Maroulakou *et al*., 1994). Two founder animals were able to transmit the C3(1)/Tag fusion gene to offspring, designated lines C and L. Both lines developed similar phenotypic abnormalities, but with different time courses which correlated to the levels of Tag produced (Maroulakou *et al*., 1994). Line C has been most extensively studied (Maroulakou *et al*., 1997; Shibata *et al*., 1996a,b; Liu *et al*., 1998).

Table 1 Incidence of foot lesion pathologic changes by age in C3(1)/Tag transgenic mice

A. Unmanipulated animals

	Age in months									
	2–3	*3–4*	*4–5*	*5–6*	*6–7*	*7–8*	*8–9*	*9–10*	*10–11*	*11–12*	*Total*
Male											
Epithelio-ecchondroma	1/1	7/8	7/9	1/1	8/8	9/9	11/11	4/4	1/1	2/2	51/57
Mixed tumor	0/1	1/8	3/9	1/1	2/8	8/9	5/11	4/4	1/1	1/2	26/57
Sarcoma	0/1	0/8	1/9	0/1	2/8	0/9	2/11	0/4	0/1	0/2	5/57
Chondrosarcoma	0/1	0/8	2/9	0/1	1/8	4/9	2/11	0/4	0/1	0/2	9/57
Basal cell carcinoma	0/1	0/8	0/9	0/1	0/8	0/9	0/11	0/4	0/1	1/2	1/57
Female											
Epithelio-ecchondroma		1/2	6/6	10/10							17/19
Mixed tumor		0/2	1/6	3/10							5/19
Sarcoma		0/2	0/6	1/10							1/19
Chondrosarcoma		0/2	0/6	0/10							0/19
Basal cell carcinoma		0/2	0/6	0/10							0/19

B. Hormone manipulated animals

	Male					*Female*			
	Intact	*Castration*	*Testosterone*	*DHT*	*Flutamide*	*Intact*	*Ovariectomy*	*Estrogen*	*Progesterone*
Epithelio-ecchondroma	51/57	10/10	6/6	12/12	5/8	17/19	11/12	18/19	13/14
Mixed tumor	26/57	8/10	2/6	5/12	5/8	5/19	3/12	6/19	3/14
Sarcoma	5/57	1/10	0/6	4/12	1/8	1/19	0/12	0/19	0/14
Chondrosarcoma	9/57	0/10	0/6	1/12	0/8	0/19	0/12	1/19	0/14
Basal cell carcinoma	1/57	0/10	1/6	0/12	0/8	0/19	0/12	0/19	0/14

Pathogenesis of foot lesions

Foot lesions were present in transgenic mice of both sexes, although, in males, the lesions were more extensive and progressed more rapidly to mixed tumors (Table 1). The frequency of development of foot lesions histologically is almost identical (30%) for both sexes at 3–6 months of age. In older male mice (7–10 months), the incidence of foot lesions increased from approximately 30–60%.

Grossly, the lesions appeared on the foot pads as firm nodules 1–3 mm in diameter (Figure 1B). Lesions were generally more extensive on the fore feet compared to the hind feet. Gross lesions were generally not apparent prior to 6 months of age.

Histologic progression of the lesions is presented in Figure 1. The eccrine sweat glands on the plantar surface of the feet in normal non-transgenic mice had low cuboidal epithelium with eosinophilic cytoplasm (Figure 1C). Ducts leading to the skin surface were

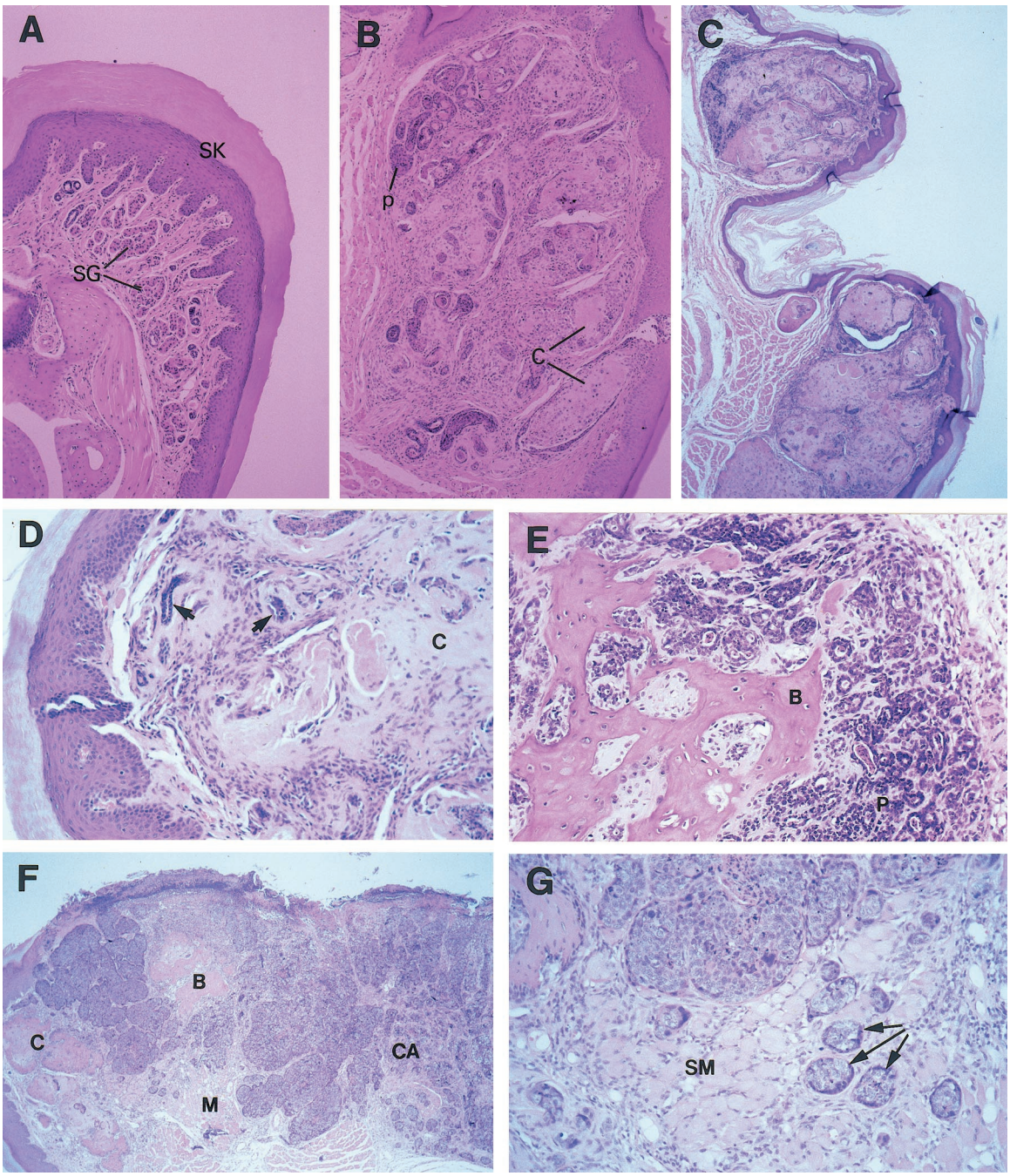

Figure 1 Development of proliferative lesions in foot pads. (**A**–**G**) histologic cross-sections of progressive lesions. (**A**) normal plantar region. SK, skin; SG, sweat gland. (**B**) lesions designated epithelio-ecchondromatosis involving proliferating epithelial cells of sweat glands (P) and surrounding mesenchymal cells with early deposition of cartilage (C). (**C**–**E**) more advanced lesions with proliferating epithelium (arrows), increased cartilage (C) and bone (B) formation. (**F**) advanced lesion with all stages of foot pad abnormalities. Cartilage (C), muscle (M), bone (B), adenocarcinoma (CA). (**G**) mixed tumor with adenocarcinoma (arrows) invading muscle (SM) and cartilage formation (C). (**A**–**G**) hematoxylin and eosin. (**A** and **B**) × 100; (**D**) × 200; (**C** and **F**) × 40; (**E** and **G**) × 400

composed of more basophilic cells. Hyperplasia of the glandular epithelium was the earliest lesion observed in the transgenic mice between 1 and 2 months of age. The cytoplasm was basophilic and decreased in volume; nuclei were closely apposed. The lumens of the glands contained inspissated eosinophilic secretions. Glands were increased in number and the basement membrane was intact. Proliferating around the glands were spindle-shaped cells (Figure 4D) set in an amorphous eosinophilic stroma (Figure 1D and F). These cells stained positively for smooth muscle actin and were myoepithelial-like (Figure 3D and E). Staining of these cells for cytokeratin was equivocal (data not shown). The matrix stained blue with Masson's trichrome stain (data not shown). These lesions enlarged and progressed as the mice aged.

In mice 3 months of age and older, the sweat gland areas lost glandular architecture, and contained ribbons of atypical, hyperplastic basal epithelial cells with minimal basophilic cytoplasm and hyperchromatic nuclei. These epithelial cells continued to proliferate in and around discrete nodules of cartilage and osteoid which appeared within these lesions. We designate such lesions containing proliferating epithelial cells and cartilage as epithelio-ecchondromatoses (Figure 1D–F).

The mixed nodular lesions of the plantar sweat glands progressed in several ways. In one type, the atypical mixed lesions expanded in size and remained well-differentiated, accompanied by proliferating basal epithelium (Figure 1E and F). A second type of progression was expandable growth of the atypical ecchondromatous nodules accompanied by overgrowth of basal epithelial cells which invaded and replaced surface epithelium and/or underlying muscle fascia and bone (Figure 1H and I). Malignant transformation occurred in some nodules which involved the cartilaginous, but usually not the osteoid, component. Occasionally, however, osteosarcomas were observed histologically and the nodular structure was obliterated by proliferating chondrocytes which invaded adjacent structures. Atypical epithelial proliferation was also associated with these lesions. Lesions that had either an invasive epithelial component (Figure 1I) and proliferation of well differentiated fibrous tissue, cartilage or bone (Figure 1H) or malignant transformation of both epithelial and mesenchymal components (Figure 4C) were diagnosed as malignant mixed tumors.

In some mice, foot lesions progressed to large fibrosarcomas with a high mitotic index. The sarcomas invaded and replaced overlying epithelium and underlying structures. The basal epithelial component was often absent in these large sarcomas. These tumors enlarged to as much as 1 cm in diameter. Metastases of the carcinomas or sarcomas were observed in less than 2% of the cases.

Early proliferative epithelial/mesenchymal type lesions were studied ultrastructurally by electron microscopy (Figure 2). They were composed of hyperplastic plantar sweat glands containing lumenal cellular debris which appeared to be composed of necrotic or apoptotic cells. The epithelium appeared hyperplastic with hyperchromatic cytoplasm and few secretory granules. The nuclei of these cells either had a normal chromatin pattern or contained prominent clumps of chromatin. The glands were surrounded by small numbers of a single layer of cells determined to be myoepithelial cells based upon their location, morphology and presence of myofibrils (Figure 2A). The mesenchymal proliferation around the glands contained fibroblast-like cells with abundant rough endoplasmic reticulum and a prominent extracellular matrix containing areas of fibrils and bundles of collagen fibers (Figure 2B). The fibroblast-like cells were found in a concentric pattern around the glands (Figure 2A). In these early lesions, cartilage-like matrix or osteoid were not seen. No epithelial to mesenchyme transition cells were observed.

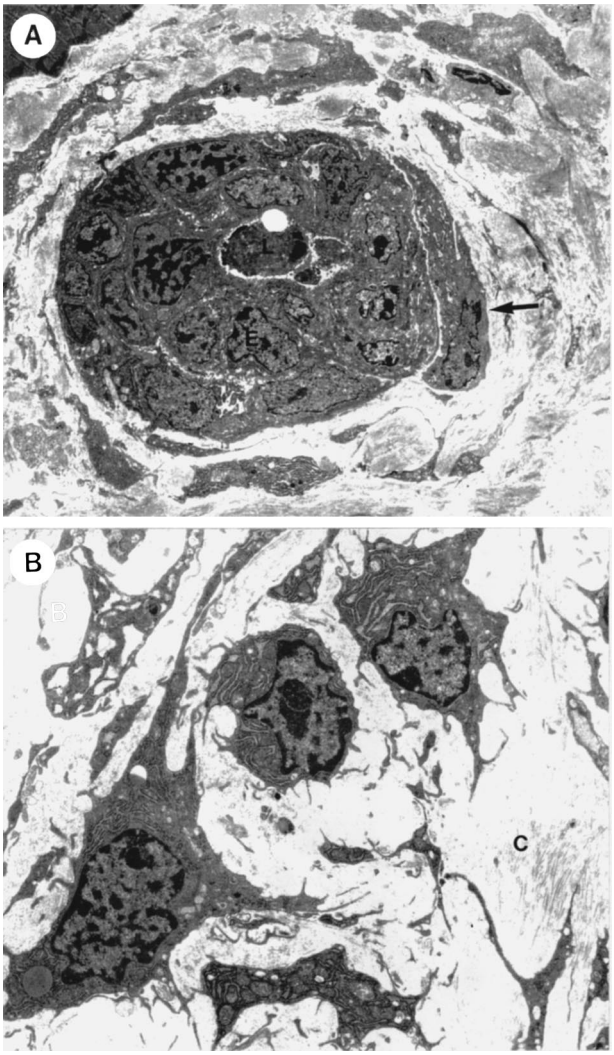

Figure 2 Electron micrographs of early sweat gland proliferative lesions. (**A**) Ultrastructure of hyperplastic plantar sweat gland contains lumenal cell debris and myoepithelial cell (arrow) surrounded by collagen matrix and portions of fibroblasts. ×3000, uranyl acetate, lead citrate. (**B**) Ultrastructure of mesenchymal proliferative lesions of plantar sweat gland showing prominent collagen fibrils (c) adjacent to fibroblast-like cells. ×9000, uranyl acetate, lead citrate

Mixed lesions in other tissues

While mixed lesions were commonly seen in the foot pads, lesions containing adenocarcinoma with cartilage were infrequently seen in other transformed tissues of the C3(1)/Tag mice including the mammary, urethral, and the salivary glands (Figure 3).

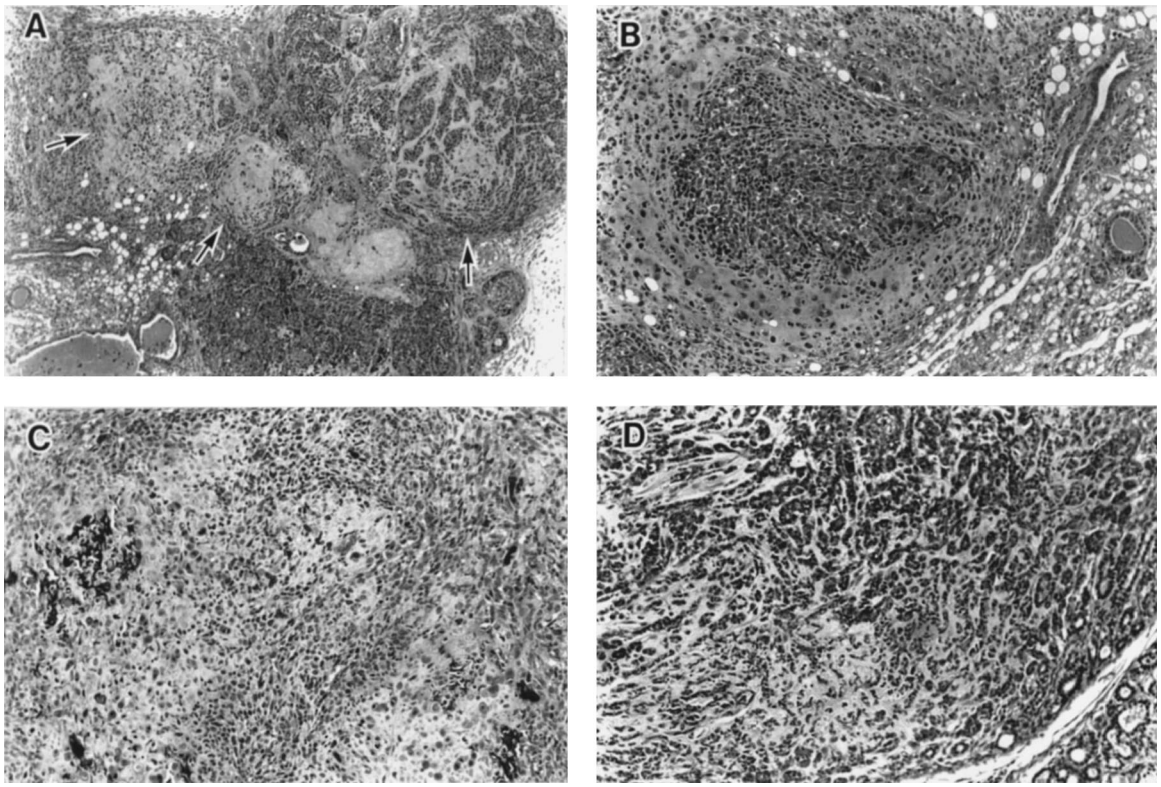

Figure 3 Mixed lesion development associated with adenocarcinomas in other tissues. Ectopic cartilage with or without bone formation was occasionally observed in association with carcinomas in the mammary gland (**A**, × 100 and **B**, × 200), urethral gland (**C**, × 200), and salivary gland (**D**, × 200)

Immunohistochemistry and special staining of foot lesions

Tag Immunohistochemical staining demonstrated that the nuclei of proliferating epithelial and mesenchymal cells, the mixed tumors and osteogenic sarcomas expressed Tag strongly. Adjacent normal tissues including the surrounding mesenchymal tissues which are not part of the sweat gland acinar unit were not immunoreactive for Tag (Figure 4A – C). Tag expression continued through all stages of mixed tumor development and was detectable in both the epithelial and mesenchymal components. The strength of the immunohistochemical reaction varied not only between tumors from the same animal, but also among cells within a single tumor (Figure 4C).

Smooth muscle actin Normal myoepithelial cells surrounding the epithelial cells of the sweat glands stained strongly positive for smooth muscle actin (Figure 4D, arrowheads) as did normal endothelial cells. Smooth muscle actin staining became greatly diminished and eventually was undetectable as the myoepithelial cells began to proliferate (Figure 1D, arrows).

Keratin Positive staining for high molecular weight cytokeratins was observed in both normal and proliferating sweat gland epithelial cells, as well as the basal cell adenocarcinomas. Staining of myoepithelial cells was equivocal whereas all mesenchymal cells were negative (Figure 4F and G).

Vimentin Vimentin staining was positive in mesenchymal cells surrounding the sweat glands but was negative in epithelial cells (Figure 4H). Sarcomatous tumors stained strongly positive for vimentin.

Met Met staining was intense in the epithelial cells forming the glandular structures of the normal plantar sweat glands (Figure 5A). The level of Met expression dramatically decreased at the earliest stages of epithelial proliferation in the foot lesions. Met expression was not observed in any mesenchymal elements including sarcomas.

TGF-beta TGF-beta1 appeared to accumulate in the extracellular matrix as the lesions progressed from early to late stages. No changes in the intracellular staining pattern of TGF-beta1 were observed. In contrast, strong staining of TGF-beta3 expression was observed in normal epithelial cells of the sweat glands (Figure 5H). However, expression of TGF-beta3 was markedly reduced as the epithelial cells began to proliferate and was not observed in the tumors of epithelial origin (Figure 5H – K).

p53 p53 nuclear accumulation directly correlated to Tag expression and was associated with the earliest stages of the developing lesions in both the epithelial and mesenchymal components (data not shown). Sustained levels of elevated p53 expression appears to occur throughout all stages of mixed tumor formation. No p53 immunoreactivity was detected in the normal epithelium or normal myoepithelium nor in nontransgenic controls.

PCNA PCNA expression, a marker of cellular proliferation (Kurki *et al*., 1986) was barely detectable in epithelial or mesenchymal cells of the normal sweat gland unit but was strongly positive in early hyperplasias as well as carcinomas and sarcomas (data not shown). PCNA expression in these lesions directly correlated to expression of Tag and p53.

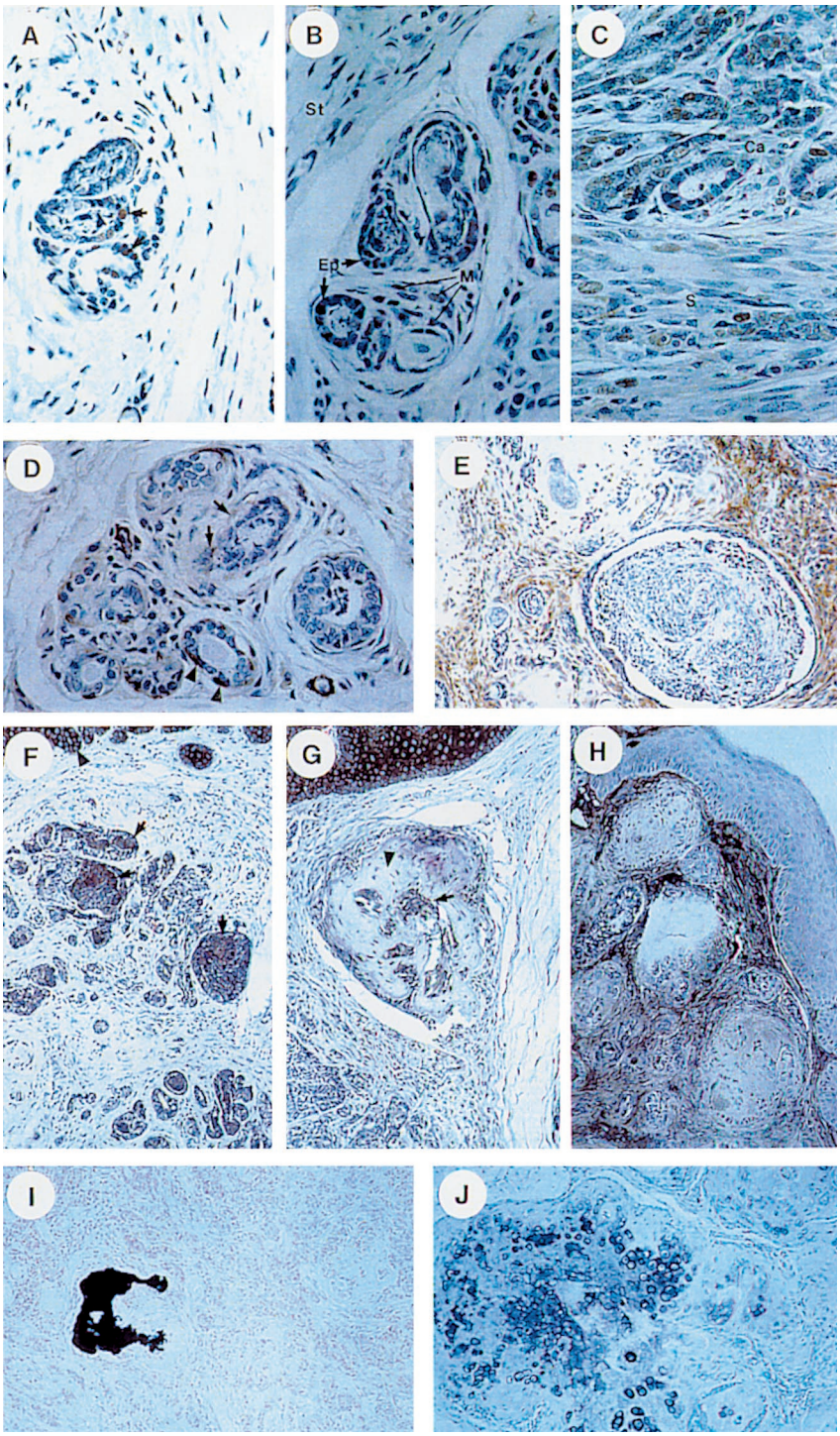

Figure 4 Immunohistochemical staining during foot lesion progression. (**A**–**C**) Tag staining. (**A**) earliest sweat gland proliferation with epithelial cells (arrows) within the acinar sweat gland unit expressing Tag. (**B**) more advanced lesion than **A** with continued Tag expression in epithelial (arrows) and myoepithelial-mesenchymal cells (arrowhead) of the acinar unit. Stromal mesenchymal cells (St) not part of the acinar sweat gland unit do not express Tag. (**C**) junction of mixed tumor with adenocarcinoma (Ca) and sarcoma (S) cells positive for Tag. (**D** and **E**) smooth muscle actin staining. (**D**) early lesion with normal myoepithelial cells (arrowheads) and proliferating myoepithelial cells (arrows) emerging from an acinar unit staining positively for smooth muscle actin. (**E**) advanced lesion with mesenchyme positive for smooth muscle actin. (**F**) early and (**G**) late lesion staining for high molecular weight cytokeratins. (**F** and **G**) proliferating epithelial cells (arrows) are positive, but (**G**) developing chondrocytes (arrowhead) and mesenchyme are negative; epidermis is strongly positive (**F**, arrowhead). (**H**) advanced lesion with strong mesenchymal staining for vimentin. (**I**) calcification in late lesion (von Kossa staining). (**J**) Cartilage matrix staining with toluidine blue. (**A**–**D**) × 600; (**E**–**I**) × 200; (**J**) × 400

von Kossa and toluidine blue von Kossa staining is useful for localizing areas of calcification. No positive staining was noted in any early lesions but was detectable only in advanced lesions where bone formation had occurred (Figure 4I). This demonstrated that the calcifications associated with endochondrial bone formation did not result from necrosis within the early lesions, but occurred as a late event in the development of the lesions. Toluidine blue staining confirmed that the developing chondrocytes laid down a cartilaginous matrix (Figure 4J).

Effect of Tabby mutation on the development of foot pad lesions

C3(1)/Tag transgenic mice were crossed with mice carying the Tabby (*Ta*) mutation, a naturally occurring genetic mutation in which sweat glands

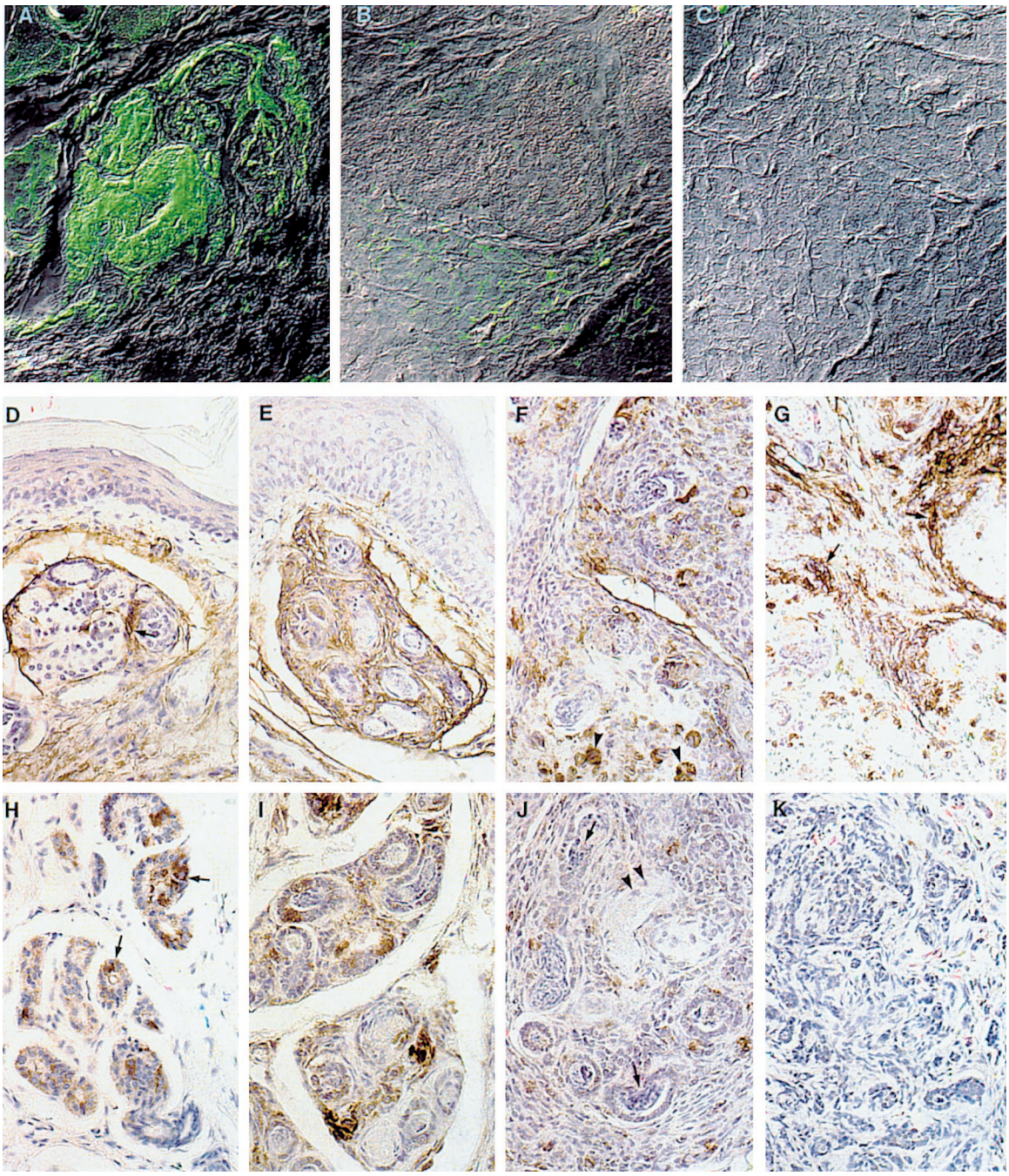

Figure 5 Analysis of Met and TGF-beta expression during foot lesion development. (**A**–**C**) immunostaining for Met in foot lesions. (**A**) normal mouse sweat glands; (**B**) proliferating epithelial cells; (**C**) mixed tumor; (**D**–**G**) epithelial extracellular TGF-beta1 around epithelial cells (arrow). (**D**) normal sweat gland epithelium. (**E**) early proliferative lesions. (**F**) epithelio-ecchondromatosis with positive staining within the proliferative epithelium and around chondrocytes (arrowheads). (**G**) advanced lesions with strong TGF-beta-1 staining in areas of proliferating epithelium. (**H**–**K**) immunostaining for TGF-beta3 during foot lesion progression. (**H**) normal sweat gland epithelium with intracellular TGF-beta3 (arrows). (**I**) early proliferative lesion with areas of reduced TGF-beta3 staining in the proliferating epithelium. (**J**) epithelio-ecchondromatosis with reduced TGF-beta3 protein in proliferating epithelium (arrow) and no staining of chondrocytes (arrowheads). (**K**) loss of TGF-beta3 staining in epithelial component of a mixed tumor. (**A**–**K**) × 600

do not develop (Blecher, 1986). Since foot pad lesions develop more prominently in male mice, only male progeny were screened for the presence of the C3(1)/Tag transgene and mice carrying the *Ta* mutation were identified by the characteristic coat color changes. Progeny carrying the C3(1)-Tag transgene but not the *Ta* mutation in the C57B6 background developed epithelio-ecchondromatoses, heterotopic bone and mixed tumors (Figure 6A, as described above for the C3(1)/tag transgenic mice in the original FVB/N background. Hybrid animals carrying both the C3(1)/Tag transgene and the *Ta* mutation, however, developed no foot pad lesions and were shown to be devoid of any sweat glands in the feet (Figure 6B). This result clearly demonstrates that the presence of the sweat gland is critical to the development of the foot pad lesions and heteropic bone.

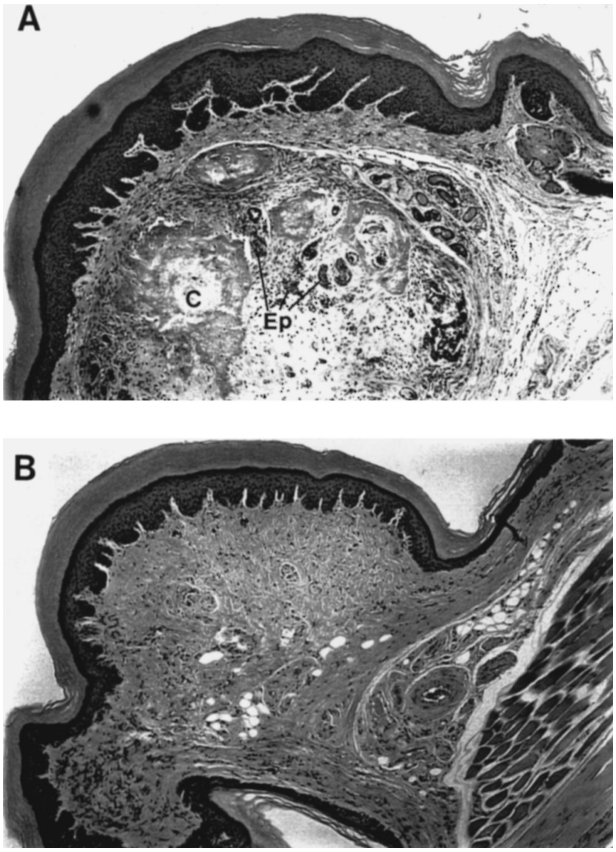

Figure 6 Effect of Tabby mutation on mixed lesion formation in the plantar sweat glands. (**A**) mixed lesion formation occurring in C3(1)/Tag transgenic mice (FVB/N × 129/SV background) but not carrying the *Ta* mutation; and (**B**) lack of sweat glands and lesion formation in the palmar foot pads of C3(1)/Tag transgenic mice carrying the *Ta* mutation (FVB/N × 129/SV background)

Effects of p53 gene dosage

To determine whether levels of p53 might be rate limiting in the development of phenotypic abnormalities, as well as tumor growth rate and progression in these transgenic animals, we examined the effect of Tag expression in combination with allelic loss of p53. The early age of death of the $p53^{-/-}$ Tag mice from malignant lymphomas precluded analysis of foot lesion formation in these mice.

Microscopic analysis of lesions from $p53^{+/+}$ or $p53^{+/-}$ mice co-expressing the C3(1)/Tag oncogene demonstrated that the histological abnormalities which developed prior to tumor formation were identical in animals of both genotypes. However, Tag male mice containing the $p53^{+/-}$ genotype developed grossly visible foot lesions at an early age compared to $p53^{+/+}$ mice. By 4 months of age only 14% (1/7) of male $p53^{+/+}$ mice were observed to have gross lesions whereas 40% (2/5) of $p53^{+/-}$ males were observed to have developed such foot lesions. The $p53^{+/-}$ male animals also developed a higher incidence of sarcomatous tumors compared to the $p53^{+/+}$ animals. Unlike the case of the male animals, a difference in the age of

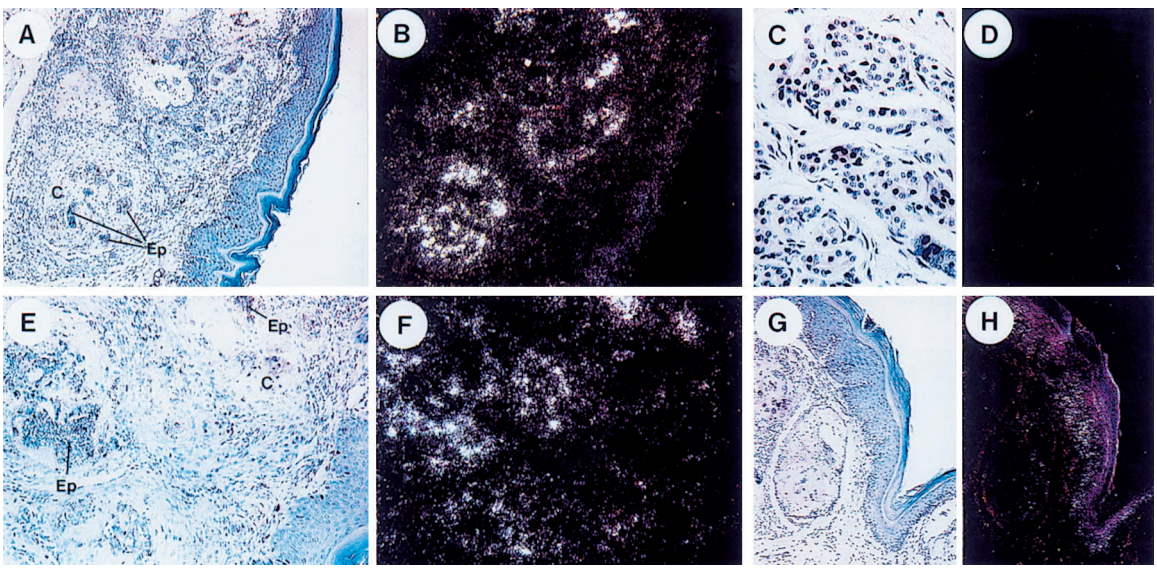

Figure 7 RNA *in situ* hybridization of foot pad lesions for BMP-2 and BMP-4 expression. (**A**–**F**) *in situ* hybridization using antisense probe for BMP-2. (**A**, **B**, **E** and **F**) C3(1)/Tag transgenic food pad lesions. (**A**) brightfield and (**B**) darkfield, ×100; (**E**) brightfield and (**F**) darkfield, ×200. (**C** and **D**) plantar foot pad from normal FVB/N male mice, ×400. (**G**) brightfield and (**H**) darkfield, *in situ* hybridization of transgenic mixed foot pad lesion using antisense probe for BMP-4

onset of lesion development between $p53^{+/+}$ and $p53^{+/-}$ females did not appear to occur. These results suggest that the presence of a mutant p53 allele in the C3(1)/Tag male transgenic mice resulted in more rapid growth of the foot lesions and the development of more sarcomatous tumors.

Hormone effects on the development of mixed tumors with bone formation

Castration or ovariectomy did not change the percentage of the animals which developed early foot lesions (Table 1B). However, significant differences in the phenotype of mixed tumor formation were observed between intact and castrated C3(1)/Tag transgenic mice. Chondrosarcomatous tumor formation was observed in 1/10 castrated mice whereas 25% of intact animals (9/57) developed such tumors. However, castrated mice that received supplemental DHT demonstrated an increased incidence of sarcoma formation (33%) compared to intact animals (9%). In contrast to the effect of androgen supplementation in castrated male mice, ovariectomy with or without high dose of estrogen supplementation did not alter the incidence of sarcoma and chondrosarcoma formation in the females. Other hormone manipulations, including low dose estrogen, high and low dose progesterone, as well as high and low dose testosterone, did not produce a significant change in the incidence of mixed tumor development compared to the intact C3(1)/Tag transgenic mice (Table 1B).

RNA in situ *hybridization for BMP family members*

In order to assess whether any BMP family members were associated with heterotopic bone formation in the foot pad lesions, RNA *in situ* hybridization was performed for expression of BMP-2, 4, 7, and 9. Sense probes for all BMP's examined were negative when hybridized to tissue sections made from foot pad lesions. However, expression of BMP-2 using the antisense probe was clearly elevated in the proliferating epithelium of the sweat glands which was associated with cartilage and heterotopic bone formation (Figure 7). No expression of BMP-2 was seen in normal sweat gland epithelium (Figure 7C and D), nor in surrounding mesenchymal elements of the foot pad lesions. Serial cut sections were negative for the other BMP's tested, including BMP-4 (Figure 7G and H).

Discussion

The C3(1)/Tag transgenic mice are a unique model system for studying the formation of heterotopic endochondrial bone associated with the development of mixed tumors. We have observed such lesions commonly arising in the sweat glands and urethral glands, and infrequently in salivary and mammary gland tumors which occur in these transgenic mice. The sweat gland lesions predictably develop over the course of several months in two transgenic founder lines which allows for the dissection of molecular events which occur during this process. Our investigations demonstrate that this process is initiated by the expression of Tag in the eccrine sweat glands of the mice (Figure 4A – C). The plantar surfaces of foot pads are the only locations where sweat glands are found in mice (Kurosomi and Kurosumi, 1970), which explains why these lesions occur only on the plantar surfaces of the foot pads and not in other areas of the skin. Tag expression in the foot pad is localized to both of the cell types in the accinar sweat gland unit, the lumen-forming epithelial cells and the surrounding single layer of myoepithelial cells (Figure 4A and B). When the C3(1)/Tag transgene is expressed in mice carrying the Tabby mutation in which sweat glands do not develop, no foot pad lesions develop (Figure 6B), confirming that the eccrine sweat glands are the source of the proliferative lesions, heterotopic bone, and mixed tumor formation in the transgenic mice.

At the earliest stage of the development of the foot lesions, both the proliferating epithelial and myoepithelial cells of the sweat gland express Tag, whereas no fibroblastic cells in the surrounding mesenchyme demonstrate Tag expression (Figure 4A and B). Both of these cell types also demonstrate high levels of PCNA and p53 proteins, which correlate to the expression of Tag (data not shown). Strong staining for the epithelial marker cytokeratin is demonstrated in both the normal and proliferating epithelial cells of the sweat glands (Figure 4F and G) but not the myoepithelial cells. Staining for smooth muscle actin, a marker for myoepithelial cells, is positive in the myoepithelial cells of the normal sweat gland (Figure 4D) but appears to be rapidly lost in the myoepithelial cells as they begin to proliferate and change morphologically from thin, fasicular cells, to larger, more rounded and elongated cells (Figure 4E). This suggests that in the early stages of the proliferative process in the sweat glands, the epithelial cells retain some degree of differentiation as determined by cytokeratin expression, whereas the myoepithelial cells become more dedifferentiated and lose the expression of smooth muscle actin.

As lesions progress through the early phase, the epithelial component becomes increasingly hyperplastic and disorganized with loss of lumen formation, although the basement membrane remains intact. The surrounding proliferative mesenchyme, originating from the myoepithelial cellular component of the sweat gland, expands between the islands of epithelial cell proliferation. That cartilage development occurred focally within the myoepithelial cells and fibroblastic zones surrounding the epithelial glandular cells suggests that the chondrocytes were derived from cells of myoepithelial origin. Thus, the epithelial and mesenchymal components in these lesions appear to arise from two separate populations of cells. As lesions progress, these myoepithelial-mesenchymal cells lay down an acid mucopolysaccharide extracellular matrix demonstrated by alcian blue and PAS staining (data not shown). These cells eventually become recognizable as chondrocytes which produce a more dense cartilage matrix as demonstrated by toluidine blue staining (Figure 4J). Most sweat gland proliferative lesions progress to this stage in which epithelial hyperplastic lesions are found adjacent to a newly formed cartilaginous component. We designate this lesion unique to the C3(1)/Tag transgenic mice as epithelio-ecchondromatosis. Ecchondromas are cartilaginous exostoses that have multicentric nonpolarized ossifica-

tion and that do not communicate with the marrow cavity. The nodular osteochondrial lesions in the foot pads are not associated with the phalanges. Atypical ecchondroma may be the most appropriate term for the mesenchymal component of these plantar nodules.

The cartilaginous matrix of the nodular lesions frequently become ossified through a process of endochondrial bone formation resulting in heterotopic bone formation. Heterotopic bone formation occurred whether the lesions remained as epithelio-ecchondromas or progress to mixed tumors. Our hypothesis regarding the developmental processes leading to the formation of mixed lesions in the transgenic mice is summarized in Figure 8. The heterotopic bone formation share some similarities to the rare human genetic condition in humans, FOP, POH, and TPO (Cohen *et al*., 1993; Kaplan *et al*., 1994; Prakash *et al*., 1989); however, the human lesions do not have an epithelial component. A recent study demonstrates that FOP is associated with the overexpression of BMP-4 by patient lymphocytes (Shafritz *et al*., 1996). The lesions in our mice do not contain inflammatory infiltrates. Other work has demonstrated that a murine stem cell chimeric model in which c-*fos* is overexpressed leads to heterotopic endochondrial bone formation, associated with the activation of the BMP-4 signal transduction pathway (Olmsted *et al*., 1998). However, while early FOP lesions overexpress BMP-4, c-*fos* does not appear to be elevated. It will be interesting to determine whether c-*fos* is elevated during lesion progression in this transgenic model. Our results show that BMP-2 and not BMP-4 is elevated during lesion development in the foot pads. This would imply that more than one osteoinductive BMP can be involved in heterotopic bone formation *in vivo*.

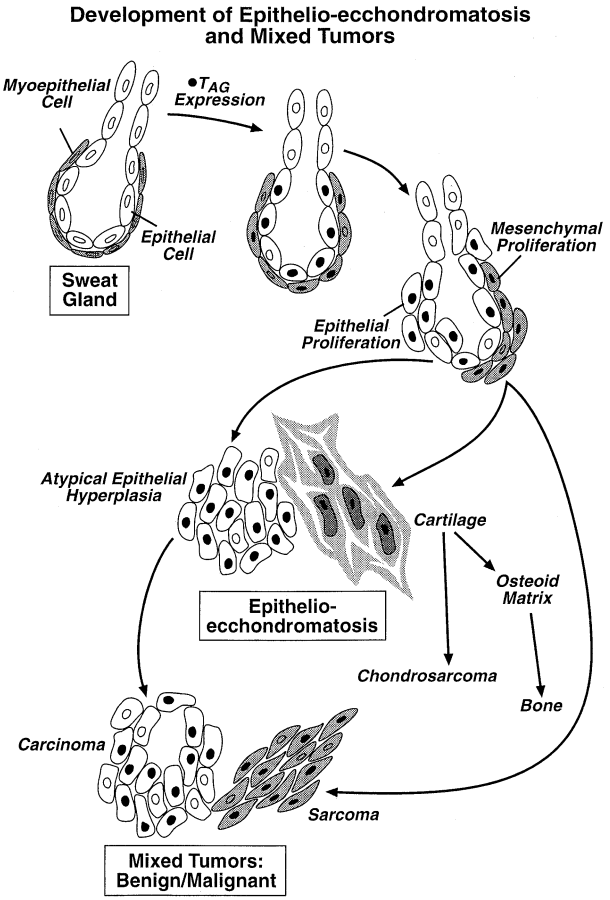

Figure 8 Diagram of foot pad lesion development leading to epithelio-ecchondromatosis and mixed tumors

The origin of cartilage and bone in mixed tumors of other glands has been controversial since the mixed tumors were first described. The controversy has centered around the relative and combined roles of connective tissue, epithelium or myoepithelium in the process of cartilage and bone formation in mixed tumors (Moulton, 1978; Pulley, 1973). Three major hypotheses have developed: (1) It has been suggested that the cartilage in mixed tumors develops from the metaplasia of epithelial cells (Huggins, 1930), possibly in conjunction with the production of paracrine factors which lead to ectopic bone formation. Whether this inductive effect is due to a diffusible factor, extracellular matrix components, cell-cell interactions, or a combination of these, has not been determined (Wozney *et al*., 1988; Rosen and Thies, 1992); (2) Some ectopic bone may develop from inducible osteogenic precursor cells, i.e. mesenchymal stem cells (Caplan, 1994), which are normally present in soft connective tissues. The identity and mechanisms of action of such inducing substances is presently not fully understood, but BMP's, members of the TGF-beta superfamily, are suspected as major factors controlling this process (Rosen and Thies, 1992); (3) Other reports suggest that the bone in mixed tumors arises by ossification of cartilage formed as the result of the metaplasia of myoepithelial cells (Pulley, 1973).

Our results are most consistent with the third hypothesis. We do not detect transitional cells arising from the proliferating epithelium and undergoing metaplasia to mesenchymal type cells. The histochemical, electron microscopic and immunocytochemical analyses of the developing foot lesions in the C3(1)/Tag transgenic mice demonstrate that the myoepithelial cells of the plantar sweat gland are the likely precursor cells of the chondrocytes, although we can not exclude the possibility that precursor cells migrate into the site and are induced to differentiate into chondrocytes. It seems quite possible that the proliferating epithelial cells induce the dedifferentiated myoepithelial cells to form heterotopic bone formation through the overexpression of BMP-2 (Figure 7).

Numerous growth factors and receptors have been shown to be critical regulators of epithelial differentiation and bone formation including members of the TGF-beta superfamily, hepatocyte growth factor/scatter factor (HGF/SCF), and the HGF receptor, met (Dean *et al*., 1985; Brinkmann *et al*., 1995). We explored whether alterations in the expression of these genes were associated with the development of the lesions containing ectopic bone. Although the epithelial cells retain expression of the differentiation marker cytokeratin, the very high level of expression of met observed in the normal epithelial cells of the sweat gland is lost as the epithelial cells become dysplastic and lose their lumenal organization (Figure 5A–C). Met expression is not observed in the epithelial tumors which arise from these lesions. Several previous studies have associated the expression of met with the ability

of epithelial cells to organize into luminal structures (Tsarfaty *et al*., 1992; Brinkmann *et al*., 1995). The results of this study are consistent with this association and strongly supports the hypothesis that met is a regulator of epithelial differentiation and lumen formation and may have tumor suppressor activity (Dean *et al*., 1985). Met staining was not observed in any of the proliferative lesions of myoepithelial/mesenchymal origin including the fibro- and chondrosarcomas (Figure 5A–C).

Paradoxical expression was noted for two members of the TGF-beta family during the progression of the foot lesions. TGF-beta1 appeared to accumulate in the extracellular matrix (Figure 5D–F). The overexpression of TGF-beta1 and its accumulation in the extracellular matrix during endochondrial bone formation has been previously reported, but its exact role in the process of bone formation remains unclear (Erlebacher *et al*., 1995). A recent study suggests that overexpression of TGF-beta1 in the arterial endothelium can cause cartilaginous metaplasia (Schulick *et al*., 1998) and endochondrial bone formation in heterotopic sites in primates (Duneas *et al*., 1998). Intracellular TGF-beta3 expression, however, was greatly reduced as the epithelial cells began to proliferate and was not observed in any tumors of epithelial origin (Figure 5G–I). Loss of intracellular TGF-beta3 expression during tumor progression suggests that TGF-beta3 might serve to inhibit epithelial proliferation and tumor formation.

Other members of the TGF-beta superfamily have been shown to play critical roles in endochondrial bone formation (Campbell and Kaplan, 1992; Rosen and Thies, 1992; Shafritz and Kaplan, 1998), and several, including BMP-2, 4, 7, 9, 10, are able to induce heterotopic bone when injected subcutaneously into mice (Erlebacher *et al*., 1995; Wozney *et al*., 1988). RNA *in situ* hybridization studies were performed on tissues during various stages of mixed tumor formation to determine the expression patterns of BMP's which are capable of inducing ectopic bone formation. Our studies indicated that the proliferating epithelial cells at early and late stages of lesion development strongly expressed only BMP-2 in association with the appearance of heterotopic bone. The overexpression of another osteoinductive morphogen, BMP-4, by lymphocytes has been associated with the early phase of FOP development in humans (Shafritz *et al*., 1996). Thus, heterotopic bone formation in human disease and this transgenic mouse model may result from the overexpression of a BMP and/or TGF-beta1 at a site which can respond to and initiate endochondrial bone formation. TGF-beta1 may synergize with other osteoinductive factors to bring about endochondrial bone formation (Ripamonti *et al*., 1997; Duneas *et al*., 1998).

Foot pad lesions developed more rapidly and with increased numbers of mixed tumors in the male animals compared to female animals. Based upon these observations, we wished to determine whether sex hormones levels directly influenced the development of the epithelio-ecchondromatosis, endochondrial bone formation or mixed tumors in the C3(1)/Tag transgenic mice. We determined that sex hormone levels do not appear to influence the formation of the early epithelio-ecchondromatosis foot lesions, but that androgens stimulate the development of the sarcomatous tumors. This conclusion is based upon the observations that: (1) the incidence of sarcomatous tumor formation is much lower in female animals compared to males; (2) castration reduces the incidence of sarcomatous tumors; and (3) castrated animals receiving DHT supplementation had a high incidence of sarcomatous tumor formation (Table 1B).

Expression of SV40 Tag in these transgenic mice may mimic genetic deficiencies of the tumor-suppressor genes p53 and Rb since Tag binds to and functionally inactivates these genes (DeCaprio *et al*., 1988; Linzer and Levine, 1979; Chen *et al*., 1992; Mietz *et al*., 1992; Ludlow, 1993). Inactivation of p53, a gene involved in cell cycle control and apoptosis (Symonds *et al*., 1994), has been observed in a majority of human neoplasms, including adenocarcinomas, chondrosarcomas, and osteosarcomas (Hollstein *et al*., 1991; Donehower and Bradley, 1993; Levine *et al*., 1994; Scholz *et al*., 1992; Dobashi *et al*., 1993; Russo *et al*., 1994; Wadayama *et al*., 1993). In this study, male C3(1)/Tag mice lacking one allele of p53 developed foot pad lesions more quickly and developed more frequent sarcomatous tumors in the foot pad. This demonstrates that despite Tag expression, p53 dosage can further affect biologic behavior of these lesions as we have recently reported for mammary tumor progression in the C3(1)/Tag transgenic mice.

These transgenic animals will advance our understanding of heterotopic bone formation associated with mixed tumor formation for which previously there has been no useful *in vivo* animal model. This transgenic mouse model is a potentially fruitful new system in which to study the cascade of events which lead to endochondrial bone formation.

Materials and methods

Transgenic animals

Derivation of transgenic animals containing the C3(1) 5′ flanking region fused to the Tag gene has previously been described (Maroulakou *et al*., 1994). Transgenic mice were maintained as heterozygotes by breeding with FVB/N nontransgenic mice (Charles River, Frederick, MD, USA). Transgenic C3(1)/Tag animals were identified by Southern and slot blot analysis of genomic tail DNA, as previously described (Maroulakou *et al*., 1994). Animals were maintained in an AAALAC-approved animal facility. Mice carrying a mutant p53 allele (Jacks *et al*., 1994) were obtained from Jackson Labs (Bar Harbor, ME, USA). Euthanasia was performed by CO_2 narcosis.

Generation of C3(1)-Tag/p53$^{+/-}$ and C3(1)-Tag/Tabby mice

Mice carrying a null mutation at the p53 locus (129/Sv background) have been described previously (Jacks *et al*., 1994). C3(1)/Tag transgenic mice containing the p53 null allele were generated through crossings of C3(1)/Tag transgenic mice with p53$^{-/-}$ mice creating a hybrid 129/Sv/FVB genetic background in the progeny. C3(1)/Tag mice homozygous or heterozygous for the p53 null allele were identified by PcR analysis (Jacks *et al*., 1994). C3(1)/Tag mice were also generated with p53$^{+/+}$ 129/Sv mice as a control for the effects of genetic background. Tabby mice (Jackson Laboratory, Bar Harbor, ME, USA), which carry an X-linked recessive mutation resulting in anhidrosis and absence of sweat glands (Blecher, 1986), were crossed

with C3(1)Tag mice to generate male mice with the following genotypes: Tag^{-}/Ta^{-}, Tag^{-}/Ta^{+}, Tag^{+}/Ta^{-}, and Tag^{+}/Ta^{+}. Animals carrying the Tabby mutation were identified by their characteristic coat color.

Histopathology

Tissues were collected, fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5 μm and were stained with hematoxylin and eosin. Selected sections were stained with alcian blue, periodic acid-Schiff (PAS), von Kossa, toluidine blue and Masson's trichrome stains.

Electron microscopy

Portions of foot lesions were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined under the electron microscope.

Immunohistochemistry

Selected lesions and normal foot pads were evaluated immunohistochemically for expression of specific antigens using one of several antibodies including high molecular weight keratins (rabbit polyclonal sera A575, dilution 1 : 100, DAKO, Carpinteria, CA, USA), SV40 Tag (mouse monoclonal, PAb 101, dilution 1 : 50, PharMingen, San Diego, CA, USA), vimentin (rabbit polyclonal M725, dilution 1 : 100, DAKO), smooth muscle actin (mouse monoclonal anti-human HHS 35, dilution 1 : 100, DAKO), p53 (mouse monoclonal Ab7, dilution 1 : 500, Oncogene Science, San Diego, CA, USA), and proliferating cell nuclear antigen [PCNA] (mouse monoclonal PC-10, dilution 1 : 400, DAKO). Unstained sections to be used for SV40 Tag, p53 and PCNA immunostaining were immersed in distilled water and heated by microwave for antigen retrieval (Shi *et al*., 1991). For keratins, unstained sections were incubated with 0.02% trypsin for 30 min at room temperature. The avidin-biotin peroxidase method was used (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as the chromagen and counterstained with hematoxylin. Met expression was determined using rabbit polyclonal anti-met C260 (dilution 1 : 50) kindly provided by Dr George Vande Woude, NCI, Frederick, MD, USA. Donkey anti-rabbit conjugated to fluorescein isothiocyanate (FITC) was used as the secondary antibody to Met and visualized by fluorescence using a Zeiss 310 LSM confocal microscope.

Hormone manipulation

Hormone effects on the development of the foot lesions in the C3(1)/Tag transgenic mice were determined by performing castration or ovariectomy with or without subsequent hormone replacement therapy. The care and treatment of experimental animals complied with NIH guidelines. Animals were anesthetized for all surgical procedures and castrations (CSX) and ovariectomies (OVX) were performed in mice at 6 weeks of age. The intact (non-surgically manipulated) and castrated or ovariectomized mice were divided into groups of ten mice each which were designated and treated as follows: Female mice: ovary intact with placebo replacement pellet (intact); ovariectomized (OVX); OVX and 17-estradiol replacement pellet with either low (physiologic) dose (1.7 mg/pellet), or high (pharmacological) dose (7.5 mg/pellet) implants; OVX and progesterone replacement pellets with either low dose (10 mg/pellet), or high dose (50 mg/pellet) implants. Male mice: intact testes with placebo replacement pellet (intact); castrated (CSX); CSX and testosterone replacement pellets with either low dose (5 mg/pellet), or high dose (25 mg/pellet) implants; CSX and dihydrotestosterone (DHT) replacement pellets with either low dose (5 mg/pellet) or high dose (25 mg/pellet) implants. Hormone supplementation was initiated 2 weeks after castration or ovariectomy by implanting pellets, which release constant amounts of hormone for 60 days (Innovative Research of America, Sarasota, FL, USA). The pellets were implanted subcutaneously with a trochar (IRA) every 60 days.

RNA in situ *hybridization*

Tissue-specific expression of several BMP's were analysed at different stages of foot lesion formation in male mice. Antisense riboprobes for mouse BMP-2, 4, 7, and 9 were hybridized with 5 micron paraffin-embedded sections as previously described (Wozney *et al*., 1993; Rosen *et al*., 1996). Sections were analysed by both brightfield and darkfield visualization using a Zeis Axioplan microscope.

Note added in proof

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human services, nor does mention of trade names, commercial products, or organization imply endorsement by the U.S. Government.

Abbreviations

BMP, bone morphogenetic protein; C3(1), rat prostatic steroid binding protein; CSX, castration; FOP, fibrodysplasia ossificans progressiva; kb, kilobase; kD, kilodalton; OVX, ovariectomy; PAS, periodic acid-Schiff stain; POH, progressive osseous heteroplasia; *Ta*, Tabby mutation; Tag, SV40 large T-antigen; TGF-β, transforming growth factor-beta; TPO, tracheopathia osteoplastica.

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